

Nonlinear Dose-Response Relationship for the Binding of the Carcinogen Benzo(a)pyrene to Rat Liver DNA *in Vivo*

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ABSTRACT

With radioactive compounds of high specific activity, the binding of carcinogens to DNA can be measured with doses that are ineffective in long-term studies. The binding of tritiated benzo(a)pyrene to liver DNA of adult male rats has been determined 50 hr after a single i.p. injection of doses between 40 $\mu\text{g}/\text{kg}$ and 4 mg/kg. The dose-response relationship is linear up to 1 mg/kg, shows a step towards 2 mg/kg, and gives a shallow linear slope above that value. The observed binding ranges from 1.7 to 180 nmoles benzo(a)pyrene per mole DNA phosphate. The nonlinearity could be due to an induction of metabolizing enzymes. The microsomal aryl hydrocarbon hydroxylase activity increases significantly 24 hr after a single dose of 4 mg/kg and 48 hr after doses of 2 and 4 mg/kg, but no induction is found with 1 mg/kg. The binding from an equimolar dose is 35 times lower than the one found on mouse skin DNA and 300 times lower than that of *N,N*-dimethylnitrosamine in rat liver. A good correlation exists to the respective tumor formation in long-term studies.

INTRODUCTION

In the currently performed long-term chemical carcinogenesis studies with laboratory animals, significant numbers of tumors are found only with concentration orders of magnitude beyond the level of human exposure. For a toxicological evaluation an extrapolation to lower doses is often done by means of different statistical approximations (9), none of which could take into account the actual course of the multiple and often dose-dependent events governing the interaction of the chemical and its metabolites with the intact animal. In this paper we show that such mathematical procedures must be interpreted with extreme caution, and we report the results of a study that could be used for reducing these uncertainties.

BP¹ is a prototype polycyclic aromatic hydrocarbon that has been shown to cause cancer in animals and that may contribute to tumors that occur in humans as a consequence of exposure to BP from a number of sources (5). By continued administration, BP can induce tumors at the site of application with only μg amounts needed per kg and day (5). Conversely, with a single dose, and for the induction of tumors distant from the site of application, much higher doses in the order of 10 mg/kg are necessary. The most frequent systemic tumors are mammary carcino-

mas in rats (14) and hepatomas in newborn mice in which the tumor incidence from a single dose of 40 mg/kg is about 20% (17).

It is now widely accepted that one possible mechanism leading to a chemically induced tumor involves binding of the chemical or one of its metabolites to DNA (11). With the use of radioactive compounds, this interaction can be measured with doses much lower than the ones used in long-term studies, and the time needed for an evaluation of a compound can be reduced to a few days. A restriction for using the reaction with nucleic acids as a short-term test (20) for carcinogenicity lies in the fact that the measured damage to the DNA does not necessarily lead to a transformation of the cell or to the growth of a tumor. Another problem is the measurement of the minute incorporation of radioactivity into DNA. In order to quantitate the low-level counts on the DNA obtained after injection of mCi amounts, all contamination must be excluded by rigorous purification of DNA and glassware (7).

We have measured the binding of [³H]BP to liver DNA of adult male rats 50 hr after a single i.p. injection of 40 $\mu\text{g}/\text{kg}$ to 4 mg/kg, thus extending another study (16) where a single dose level of 1.2 mg/kg was used.

MATERIALS AND METHODS

Chemicals. Inactive BP was obtained from Fluka AG, Buchs, Switzerland. Generally tritiated BP was from the Radiochemical Centre, Amersham, England, in 2 batches with 3.4 and 36 Ci/mmol, respectively. BP was purified by column chromatography in hexane on silica gel. NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, RNase A, and trypsin were from Boehringer/Mannheim, Mannheim, Germany. Bovine serum albumin (Fraction V) was from Sigma Chemical Co., St. Louis, Mo. 3-Hydroxybenzo(a)pyrene was a gift from the National Cancer Institute Chemical Repository at IIT Research Institute, Chicago, Ill. Insta-Gel and Soluene 100 were from Packard Instrument Co., Downers Grove, Ill. All other reagents used were of the purest grade available from Merck AG, Darmstadt, Germany.

Animals. Male rats (SIV-50, Sprague-Dawley derived, 260 to 340 g) were obtained from the Kantonales Tierspital, Zürich, Switzerland, and were housed 1/cage. Nafag laboratory chow (Nafag AG, Gossau, Switzerland) and tap water were provided *ad libitum*. At 9 a.m. they were given an i.p. injection of BP in 0.5 ml sunflower seed oil with a total radioactivity of 0.5 to 1 mCi, diluted, if necessary, with inactive BP to the dose required. For the measurement of AHH activity, inactive BP was used.

Isolation of DNA. Fifty hr after the injection, the liver was excised under ether anesthesia, frozen over liquid nitrogen,

¹ The abbreviations used are: BP, benzo(a)pyrene; AHH, aryl hydrocarbon hydroxylase; sucrose-TKM, 0.25 M sucrose in 50 mM Tris-HCl (pH 7.5); 2.5 mM KCl; 5 mM MgCl₂.

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and stored at -20° until further use. The large liver lobe was treated according to the method of Markov and Ivanov (10) by a method yielding a DNA contaminated with less than 1% RNA and 0.1% protein. As a modification to their Procedure 1, a custom-made, air-tight aluminum headpiece on a Waring Blendor was used for the homogenization, and the chloroform:phenol extraction was performed with only one-third of the aqueous volume. The subsequent phase separation was achieved with 15 min centrifugation at $20,000 \times g$ on a Sorvall SS-3 centrifuge (DuPont Instruments, Newtown, Conn.) at room temperature.

After the final purification step, 1 to 2 mg dried DNA were redissolved in 0.014 M sodium phosphate buffer, pH 6.8, and the radioactivity was counted in a BF 5000 scintillation counter (Laboratorium Dr. R., Berthold, Wildbad, Germany) after the addition of 10 ml Insta-Gel. The net counts ranged from 15 to 250 cpm. Treatment of this DNA with RNase and trypsin did not reduce the specific activity, nor did repeated washing with ethanol:ether (2:1). The amount of DNA was determined by UV analysis by assuming an absorbance of 19 for a solution of 1 mg DNA per ml at 260 nm and pH 6.8.

Incorporation of Tritium from Tritiated Water into DNA. Administration p.o. of 1 mCi tritiated water to 300-g rats resulted in a specific activity of urine and body water of about 10^7 dpm/ml. The incorporation of radioactivity into DNA from such an exposure resulted in 13 dpm/mg after 24 hr.

Radioactivity in Whole Blood. Fifty μ l heparinized blood from the tail vein were treated with 1 ml Soluene 100:isopropyl alcohol (1:1) for 10 min at room temperature, 0.5 ml 30% H_2O_2 was added, and the mixture was held at 40° for 20 min. The sample was counted after the addition of 10 ml Insta-Gel:0.5 M HCl (9:1).

Preparation of the Liver Microsomes. All manipulations and centrifugations were performed at $3-5^{\circ}$. The animals were killed by stunning and cervical dislocation, the liver was excised and washed twice in ice-cold sucrose-TKM. Four g of liver from the large lobe were homogenized in 12 ml 0.25 M sucrose-TKM in a loose-fitting Potter-Elvehjem homogenizer with a Teflon pestle of 0.2-mm clearance with 10 up-and-down strokes at 500 rpm. The homogenate was centrifuged at $2,000 \times g$ for 20 min in a Sorvall RC-5B centrifuge, and the supernatant was used for the preparation of microsomes according to the Ca^{2+} precipitation method (18). It was centrifuged for 10 min at $12,000 \times g$, and an aliquot of the supernatant was rapidly mixed with 5 volumes of a solution containing 10 mM sucrose and 9.6 mM $CaCl_2$. The tube was allowed to stand in ice for 10 min and centrifuged for 8 min at $1,000 \times g$. The pellet was resuspended in the original volume of the aliquot with 0.25 M sucrose-TKM, and the same step was repeated. The final pellet was resuspended in 2 ml of the AHH assay buffer.

AHH Assay. Reactions were carried out in 25-ml wide-neck conical flasks at 37° . All reagents except BP were prepared in 50 mM Tris-HCl, pH 7.5:3 mM $MgCl_2$:1 mM KCl (assay buffer). To 1 flask were added: 800 μ l of the assay buffer; 100 μ l of a cofactor mixture containing glucose 6-phosphate (57 mg/ml); NADP (32 mg/ml); glucose-6-phosphate dehydrogenase (40 units/ml; 40 μ g/ml); and 50 μ l of bovine serum albumin (40 mg/ml), which enhances the

AHH activity (1) and gives a better linear relationship between protein concentration and AHH activity. One ml microsomal suspension in assay buffer containing around 200 μ g protein was added, and the flasks were preincubated for 5 min in a shaking incubator. The reaction was started with the addition of 50 μ l of an 808- μ g/ml BP solution in acetone. At 3 and 6 min after the addition of BP, 0.5 ml of the incubation mixture was placed in a Teflon-capped tube in ice, containing 375 μ l acetone and 125 μ l 1 N HCl. Two ml hexane were added, and the tube was warmed up to 37° and vigorously shaken for 10 min at room temperature. One ml of the organic phase was extracted similarly with 3.5 ml 1 N NaOH. The fluorescence of the aqueous phase was measured immediately in a Perkin Elmer 203 fluorescence spectrophotometer with a 10-nm bandpass at 396 nm (excitation) and 522 nm (emission). A quinine sulfate solution (1 μ g/ml in 50 mM H_2SO_4) was used as fluorescent standard to calibrate the fluorometer. Standard solutions of synthetic 3-hydroxybenzo(a)pyrene in 1 N NaOH were used to check the accuracy of the fluorometer. The determinations were performed in duplicate for each animal.

Protein Measurements. These were made in duplicate according to the method of Lowry et al. (6) with the modification that the solutions were made 0.1% in sodium dodecyl sulfate.

RESULTS

The binding of BP to liver DNA as a function of the dose administered is depicted in Chart 1. It shows a linear rise up to approximately 1 mg/kg, a step towards 2 mg/kg, and a shallow slope above that value. The points were divided into groups of 14 and 11 values below and above the presumed step. The linear regressions calculated from the 2 groups were extrapolated to the step region and the standard deviations were calculated. Since there is no overlap of the 2 S.D. bars, the step is statistically significant.

The radioactivity measured on the DNA could be due to a covalent binding of a BP metabolite, to a nonenzymatic, intercalative binding, or to the incorporation of tritium by regular biochemical pathways from tritiated water formed by oxidative metabolism of $[^3H]BP$. For determination of the contribution from the tritiated water, its specific activity in urine was determined 24 hr after an i.p. injection of 1

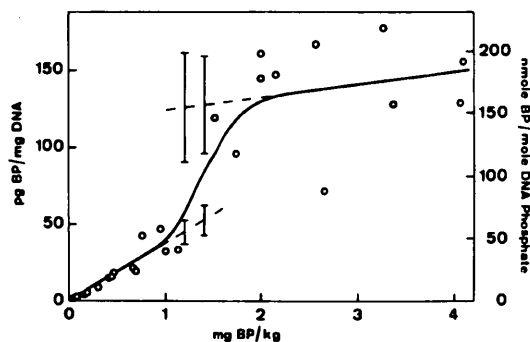


Chart 1. Binding of $[^3H]BP$ to rat liver DNA 50 hr after single i.p. injection, as a function of the dose administered. Each circle represents 1 animal. Bars at 1.2 and 1.4 mg/kg cover 2 S.D. calculated from the 2 regressions with the values below and above the nonlinearity.

mCi [³H]BP. It amounted to about 3×10^5 dpm/ml which, on the basis of the control experiment, would give rise to negligible 0.4 dpm/mg DNA in 24 hr.

Noncovalently bound BP is almost completely removed during the isolation of DNA. Less than 2 cpm/mg DNA were measured after the incubation of 0.1 mCi [³H]BP with the denatured homogenate of 4 g liver for 2 hr at room temperature. We therefore conclude that the radioactivity on the DNA from the main experiments reflects the covalent binding of BP metabolites that are known to retain their tritium label completely (13).

The radioactivity in whole blood showed a peak level 22 hr after the i.p. injection of BP and decreased to about two-thirds of that value by 50 hr. The corresponding concentration of BP plus metabolites was proportional to the dose administered and amounted to 120 ng/ml from an injection of 500 μ g/kg, at the time of the peak level.

It is well known that metabolic activation of BP is required before a metabolite can undergo a covalent interaction with other molecules (19). The enzyme system that is responsible for this activation, the AHH (BP hydroxylase, EC 1.14.14.2.), is inducible by many chemicals, including BP. A single i.p. BP dose of 2 mg/kg to 40- to 60-g rats caused a 2-fold increase of AHH activity in liver homogenate 3 hr after the administration and a 5-fold increase after 12 hr (3). The nonlinearity of our dose-response relationship lies in that dose range so that the step could be due to an induction of this activating enzyme system.

In order to check this hypothesis, we measured the microsomal AHH activity 24 and 48 hr after i.p. doses of 1, 2, and 4 mg/kg with rats of the same strain and age as the ones used in the binding study. Table 1 shows that there is in fact a significant induction from 4 mg/kg after 24 hr, and from 2 and 4 mg/kg after 48 hr, whereas from 1 mg/kg no significant effect can be observed.

DISCUSSION

We have demonstrated a nonlinear dose-response relationship for the binding of BP to rat liver DNA *in vivo* and presented evidence that the nonlinearity between 1 and 2 mg/kg could be due to the microsomal activity that is

induced in the high dose range only. Between 2 and 4 mg/kg, the induction is further increased, while the binding is only slightly enhanced. A saturation of the blood from the higher doses must be excluded as an explanation of that shallow slope, since we have found that the concentration of BP metabolites in whole blood, 6 hr after the injection, increases linearly with the dose. It therefore seems that the microsomal AHH activity is not the only dose-dependent modulator of the binding of BP to DNA. Other, unknown parameters, such as limitations in the formation or transport of the reactive metabolite or an induction of repair mechanisms or detoxifying enzyme systems, must also play a role with higher doses.

The intake of BP by humans is strongly dependent on the living conditions and ranges in the order of 0.1 μ g/kg/day (15). A purely mathematical extrapolation of the tumor incidence from the high doses needed in long-term tests to these human exposure levels would never have predicted a step like the one found in our experiments. Such a dose-effect study could therefore help to improve the extrapolation of carcinogenicity data to low doses in a biologically founded way. In addition, our dose-response curve could give one possible explanation for synergistic effects in chemical carcinogenesis. Since the activating enzyme system is inducible not only by BP itself but also by a variety of ubiquitous compounds, e.g., pesticides, the binding from a given BP dose could be enhanced as compared to an unexposed individual.

The amount of BP bound *in vivo* to DNA's of various organs correlates well with the respective tumor formation. BP acts predominantly at the site of administration and a single painting of mouse skin with 1 mg BP per kg yields 1700 nmoles BP bound per mole DNA phosphate (2, 4), i.e., about 35 times the binding in liver from an equal i.p. dose. Repeated painting with this dose is strongly carcinogenic for mouse skin, whereas no tumors are induced in the liver with daily p.o. doses of even 2.5 mg/kg (5). Conversely, with the typical liver carcinogen *N,N*-dimethylnitrosamine, chronic feeding with 2 mg/kg leads to liver tumors in most of the animals (8) and a single i.p. injection of this dose produces 150 μ moles alkylations per mole DNA phosphate (12), 300 times more than an equimolar dose of BP would generate.

This quantitative discussion of binding and carcinogenesis is still very speculative, since, among other problems, the mutagenicity of different types of damages on the DNA, the efficiency of the corresponding repair mechanisms, and the cell division rates are not equal for the examples given. Nevertheless, such an approach will be helpful for extrapolations of long-term carcinogenicity data to low doses as well as for a refined assessment of the carcinogenic power of different chemicals.

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Table 1
Liver microsomal AHH activity in adult male rats 24 and 48 hr after a single i.p. injection of 1, 2, and 4 mg/kg

Dose (mg/kg)	24 hr		48 hr	
	AHH units ^a	Induction relative to control	AHH units	Induction relative to control
0 (control)	1434 \pm 109 ^b		1632 \pm 240	
1	1701 \pm 249	1.19	1529 \pm 173	0.94
2	1843 \pm 437	1.28	2123 \pm 231	1.30 ^c
4	2514 \pm 676	1.75 ^c	2440 \pm 9	1.50 ^c

^a One AHH unit is expressed as the amount of enzyme that catalyzes the formation of alkali-extractable products in 1 min per mg protein causing a fluorescence equivalent to that of 1 pmole synthetic 3-hydroxybenzo(a)pyrene.

^b Mean \pm S.D. ($n = 3$).

^c Significantly different from control (Student's *t* test, 1-sided, $p < 0.05$).

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